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Complement Fixation by Pemphigus Antibody. I. In Vitro Fixation to Organ and Tissue Culture Skin

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Although complement is often detected in the intercellular substance of pemphigus skin lesions, the ability of pemphigus antibodies to fix complement in vitro is controversial. The purpose of this study was to test in vitro complement fixation abilities of pemphigus antibodies further using organ and tissue culture methods. Epidermal cell monolayers from mouse tail were incubated with the purified IgG fraction of pemphigus serum followed by purified Clq. Binding of Clq, as well as IgG was demonstrated by immunofluorescence methods. When purified Clq was replaced with normal human serum as a complement source, positive C3 and C4 staining were also evident. When purified IgG of normal human serum was used in place of pemphigus IgG, similar immunofluorescence staining was not observed. Further evidence for complement fixation in vitro by pemphigus antibodies was obtained using organ cultures. Organ culture of normal human skin and monkey esophageal mucosa cultured in purified pemphigus IgG showed intercellular substance binding of IgG. No binding was observed when normal IgG was substituted for pemphigus IgG. Additional organ culture sections were then treated with complement (fresh normal human serum) and tested by in vitro complement staining. Fixation of Clq, C4, and C3 was noted in intercellular substance areas of organ cultured skin and mucosa incubated with pemphigus IgG but not those incubated with normal IgG. Prior treatment of pemphigus IgG organ cultured skin sections with unlabeled anti-C3, blocked positive C3 staining. These results suggest that some pemphigus antibodies are capable of activating complement in vitro.

Previous studies have implicated the complement system in the pathogenesis of a number of bullous skin diseases including bullous pemphigoid, cicatricial pemphigoid, and herpes gestationis [1]. Despite the presence of IgG class autoantibodies reactive with an intercellular substance (ICS) of skin and mucosa in serum samples of patients with pemphigus [2,3], the ability of these antibodies for activating the complement system has remained controversial. In an early study, Jordon et al [4] failed to demonstrate that pemphigus antibodies would fix complement despite the fact that they are found in IgG subclasses normally associated with complement activation [5] and the fact that complement deposits are routinely found in acantholytic areas of pemphigus skin lesions by direct immunofluorescence (IF) staining [6–8]. Later, Nishikawa and coworkers [9,10] were able to show that some pemphigus sera would fix complement to ICS areas of skin using in vitro C3 IF staining. As a variety of conflicting theories have evolved to explain Nishikawa's findings, including participation of other serum factors, we undertook the present studies in an attempt to resolve the issue. Using organ and tissue culture methods, which mimic acantholysis in vitro, and purified IgG fractions, we have tested the complement-fixing abilities of pemphigus antibodies further.

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Abbreviations:
FITC: fluorescein isothiocyanate
HBSS: Hanks' balanced salt solution
ICS: intercellular substance
IF: immunofluorescence
IgG: gamma globulin
NHS: normal human serum
VBS++: veronal-buffered saline containing 0.5 mM magnesium chloride and 0.15 mM calcium chloride

MATERIALS AND METHODS

Immunoglobulin Fractions

Sera from 2 patients with pemphigus vulgaris were obtained by plasmapheresis. These patients had pemphigus antibody titers of 640 (patient 1) and 160 (patient 2) using monkey esophagus as tissue substrate. Serum from a healthy donor and pooled normal human serum (NHS) (Gibco Laboratories, Grand Island, New York) served as controls. IgG purification of heat-inactivated normal and patient serum (56°C, 30 min) was performed by precipitation with 50% saturated ammonium sulfate followed by chromatography of DEAE-cellulose (DE52, Whatman, Clifton, New Jersey) with 0.01 M Tris buffer at pH 8.0. The fractions of the first peak were collected, dialyzed against 3 changes of distilled H_2O at 4°C, lyophilized, and stored at −20°C.

Sources of Complement

Fresh NHS was obtained from a healthy individual with AB blood type for complement binding studies in organ culture, and for the absorbed NHS used in the tissue culture. Absorption of NHS was carried out by the following step to remove antibody to substances of mouse epidermal cell surface. The cells obtained from mouse tails were suspended in 2 ml of fresh NHS at 37°C for 2 h and incubated at 4°C overnight with gentle shaking. After centrifugation at 2000 rpm for 20 min, the absorbed serum from the supernate was collected and stored at −70°C.

By hemolytic complement assay, the CH_{50} of absorbed NHS was 52 units. C2-deficient serum used in control studies was also absorbed in a similar fashion.

Clq was purified from fresh NHS according to the method described by Yonemasu and Stroud [11] with minor modifications [12]. Purity was checked by double immunodiffusion (Ouchterlony); protein concentration was estimated by reading on a spectrophotometer at 280 nm. Purified Clq was also used as a source of complement in some of our studies.

Antiserum

Unlabeled antiserum to C3 component of complement was prepared by the methods previously outlined in detail [13]. Monospecific antiserum to human IgG was prepared by injecting goats with a DEAE cellulose purified gamma globulin (IgG), followed by purification by affinity chromatography using human IgG coupled to Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The monospecific antiserum was then conjugated with fluorescein isothiocyanate (FITC). FITC-labeled goat antihuman Clq, C4, and C3 serum were purchased (Meloy Laboratories, Inc., Springfield, Virginia) and tested for sensitivity by previously described methods [8,13].

Epidermal Keratinocyte Culture

Epidermal cells from adult mouse were cultured using a modification of the technique of Brysk et al [14]. Briefly, the tail skin removed from adult mouse (C3H, DBA/2) was placed for 1 h in an antibiotic solution containing 0.1 mg/ml gentamycin sulfate (GRS Garamycin, Schering Co., Kenilworth, New Jersey), 75 U/ml mycostatin (Gibco Laboratories), and 400 IU penicillin-400 mcg streptomycin/ml (Flow Laboratories, McLean, Virginia) in Hanks' balanced salt solution (HBSS) (Flow Laboratories) without Mg++ or Ca++. The tissue was then incubated in 0.5% trypsin in HBSS at 37°C for 1 h, and the dermis and epidermis were separated and processed by gentle teasing. The suspension was filtered through sterile gauze to eliminate skin and hair fragments and centrifuged at 1000 rpm for 10 min. The pellet was resuspended in 0.002% DNase I (Sigma Chemical Co., St. Louis, Missouri) in Medium 199 (modified) without calcium chloride (Flow Laboratories) for 1 h at room temperature, and separated on a discontinuous Percoll (Pharmacia Fine Chemicals, Inc.) gradient (1.02, 1.04, 1.06, 1.07, 1.09 g/cm^3) (2500 rpm, 20 min). Cells were collected from the 1.07–1.09 interface of the tube. After washing to remove Percoll, the cells were resuspended in Eagle's minimum essential medium with HBSS and Hepes (MA Bioproducts, Walkersville, Maryland) containing 10% fetal bovine serum (MA Bioproducts), 0.292 mg/ml L-glutamine (Gibco Laboratories), 200 U penicillin–200 mcg streptomycin/ml, and 0.03 mg/ml gentamycin and then plated in collagen (Vitrogen 100, Flow Laboratories)-coated tissue culture chamber/slides (Miles Laboratories, Napersville, Illinois). The cells were cultured in 5% CO_2 at 37°C. The medium was changed every 3 days.

Organ Culture

Organ culture was carried out by the method of Sarkany et al [15] with modifications described by Schiltz and coworkers [16]. Specimens of adult human skin from patients undergoing amputation, or monkey esophagus were cut to a thickness of 0.3 mm with Castroviejo electrokeratome (Storz Surgical Instruments, St. Louis, Missouri). The sheets were cut into squares (approximately 2 × 2 mm), and a single square of skin was placed dermal side down on a larger square of sterile lens paper (approximately 1 cm^2). The edges of the paper were dipped into molten paraffin wax. The paper rafts were floated on the surface of the appropriate culture medium in culture dishes and placed in a humid atmosphere containing 5% CO_2 in air at 37°C. Control culture media consisted of heat-inactivated pooled NHS (Gibco), while test media consisted of heat-inactivated pooled NHS containing pemphigus IgG to a final concentration of 6.75 mg/ml (IF titer 1:128 patient 1, 1:32 patient 2 when tested on monkey esophagus). Cultured skin was removed after 48 h of incubation and was quick-frozen in liquid nitrogen and stored at −70°C.

Immunofluorescence Procedures

Epidermal cell monolayers (5 days after plating) from mouse tails were incubated in phosphate-buffered saline containing 3 mg/ml of pemphigus IgG (IF titer 1:80 patient 1, 1:20 patient 2 when tested on monkey esophagus) or normal IgG. Then the cells were incubated with either 25 mg/ml of purified Clq dissolved in veronal-buffered saline containing 0.5 mM magnesium chloride and 0.15 mM calcium chloride (VBS++), or absorbed NHS diluted 1:10 in VBS++. As controls heat-inactivated NHS at 56°C, 30 min, 15 mM Mg++-EGTA or 10 mM EDTA-treated NHS were used in place of these complement sources. Additional control was the substitution of C2-deficient serum diluted 1:10 in VBS++ which was absorbed on epidermal cells. IgG and complement binding were examined using FITC-goat antihuman IgG, Clq, C4, and C3 antisera. In addition, blocking of C3 staining was accomplished by prior incubation with unlabeled goat antihuman C3 serum before IF staining.

Organ culture specimens stored at −70°C were cryostat sectioned at 4 μm, mounted on glass slides, and air dried for 30 min. Slides were then incubated with fresh NHS or C2-deficient serum diluted 1:10 in VBS++. Controls were performed using heat-inactivated EDTA or Mg++–EGTA-treated NHS. IgG and complement binding were detected using the above-mentioned conjugated antisera.

Pemphigus and normal IgG fractions were also tested for fixation of C3 using cryostat sections of adult normal human skin and monkey esophagus by in vitro complement staining.

RESULTS

Treatment of cryostat sections of either normal human skin or monkey esophagus with the IgG fraction from pemphigus patient 1 resulted in positive C3 staining of the ICS. Similar, but minimal ICS staining was observed using the IgG fraction from pemphigus patient 2, whereas no staining was observed using the normal IgG fraction. The positive ICS staining reactions observed using pemphigus IgG fractions on these normal tissues, however, was less intense than the staining reactions which resulted from the procedures described below.

Mouse epidermal cells covered approximately 80% of the surface of the culture slide by day 3, and a complete monolayer was formed by day 5. By direct IF staining, the monolayer cells treated with pemphigus IgG from patient 1 showed granular staining (speckled pattern) of the cell surface when treated with FITC-labeled antihuman IgG. When purified Clq was added as a source of complement, staining with a similar speckled pattern was noted (Fig 1*a*) by in vitro Clq IF staining. When purified Clq was replaced by absorbed NHS as the source of complement, C3 and C4 as well as Clq binding were observed (Fig 1*b*) by in vitro Clq, C3, and C4 staining. On the other hand, neither IgG nor complement (Clq, C4, and C3) binding was observed on cell surfaces treated with IgG fractions of NHS (fig 1*c*). When cultured epidermal cells were treated with the IgG fraction of pemphigus patient 2 and tested for Clq, C4, and C3 binding in a similar manner, the staining patterns observed were identical to those obtained with patient 1. The staining, however, was less intense.

To determine the mode of fixation of complement by pemphigus IgG, selected control tests were performed using the IgG fraction from patient 1 (Table I). As noted previously, positive Clq, C4, and C3 staining occurred when NHS was used as the source of complement, whereas only positive Clq staining was observed when purified Clq was used as the complement source. Heat inactivation of the complement source resulted in inhibition of all 3 complement staining reactions. Treatment of the complement source with EDTA decreased the intensity of Clq staining while completely inhibiting both C4 and C3 staining. When Mg++–EGTA was added to the complement source, partial inhibition of Clq and C4 staining occurred, while C3 staining was again completely inhibited. Partial inhibition of the Clq and C4 staining reactions, when chelators are added to the complement source, is consistent with our previous observations [17,18]. Substitution of C2-deficient serum as the source of complement also yielded negative C3 staining but positive Clq and C4 staining (Fig 2). In addition, prior treatment with unlabeled anti-C3 antiserum blocked positive C3 staining.

Further evidence for complement fixation in vitro by pemphigus IgG was obtained using organ cultured skin specimens. IgG binding was observed in the ICS areas of both human skin and monkey esophageal organ culture specimens after a 48-h cultivation with the pemphigus IgG fractions. At 48 h, minimal or early evidence of acantholysis was apparent in the specimens. Clq, C4, and C3 staining reactions were noted in the ICS areas of these explants when cryostat sections were treated with complement (fresh NHS) and labeled antisera to Clq, C4, and C3 (Fig 3). The same control reaction patterns, as described for the cultured mouse epidermal cells, were noted when the sections of organ cultured human skin were treated with C2-deficient serum, heat-inactivated NHS, or NHS treated with either EDTA or Mg++–EGTA. Prior treatment of the complement-treated organ cultured skin sections with unlabeled anti-C3 resulted in blocking of the positive C3 staining. When organ cultures were cultivated with normal IgG, rather than with pemphigus IgG, no positive complement staining reactions were noted.

DISCUSSION

As noted earlier, the ability of pemphigus antibodies to fix complement has been the subject of considerable debate. For many years, these antibodies were thought to be non-complement fixing. The fact that complement components are rou-

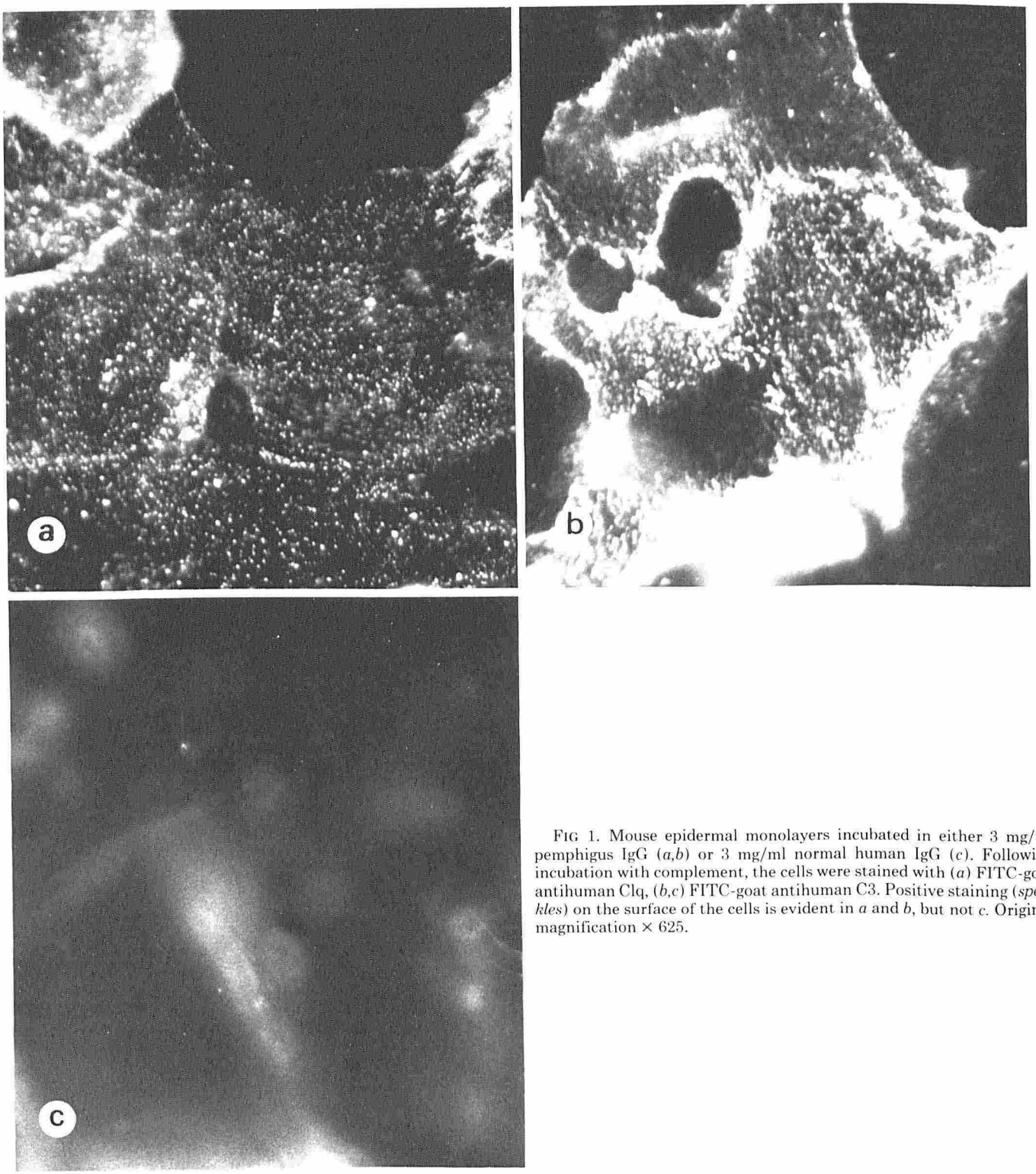

FIG 1. Mouse epidermal monolayers incubated in either 3 mg/ml pemphigus IgG (*a,b*) or 3 mg/ml normal human IgG (*c*). Following incubation with complement, the cells were stained with (*a*) FITC-goat antihuman Clq, (*b,c*) FITC-goat antihuman C3. Positive staining (*speckles*) on the surface of the cells is evident in *a* and *b*, but not *c*. Original magnification × 625.

tinely found deposited in pemphigus skin lesions led Nishikawa, Hashimoto, and coworkers [9,10] to reinvestigate this phenomenon further some years later. Their positive findings, however, have only led to more controversy, leading some to suggest that other serum factors, such as rheumatoid factors, might account for the observed complement fixation.

In an attempt to resolve this controversy, we chose to use purified IgG fractions of both pemphigus and normal human serum. Since heavy deposition of various complement components is seen in areas of acantholysis but not in normal-appearing areas of pemphigus skin [6–8], we also chose to use organ and tissue culture skin cells in these experiments in an attempt to mimic acantholysis in vitro. Our contention was that in areas of early-forming acantholysis, where cells are beginning to separate, more efficient fixation of complement might take place. Although some fixation of complement by pemphigus antibody was observed using normal substrates (normal human skin and monkey esophagus), the staining was less intense than the staining of cultured epidermal cells, and organ cultured skin partially supporting this contention. Our positive findings also support the reports of Nishikawa et al [9] and Hashimoto et al [10] suggesting that at least some pem-

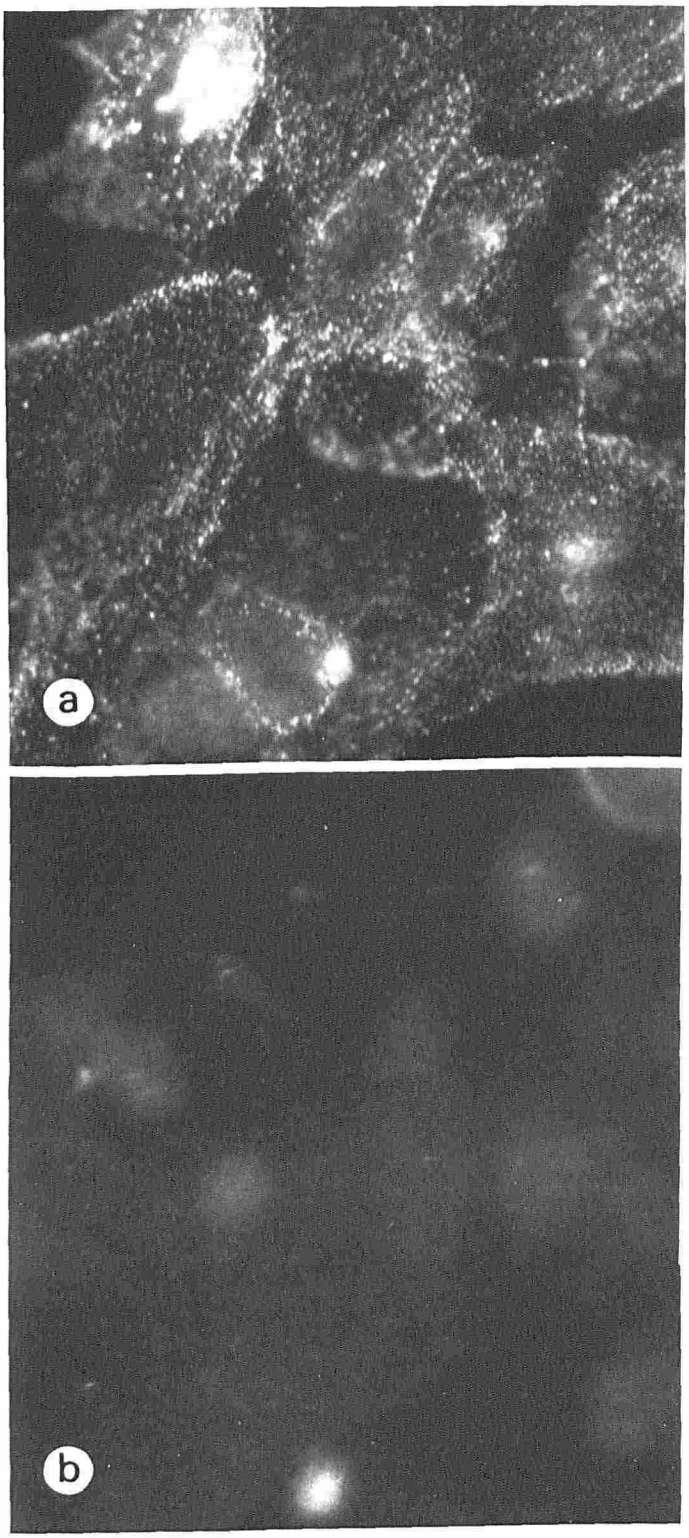

FIG 2. Mouse epidermal monolayers incubated in 3 mg/ml pemphigus IgG. The cells were then incubated in C2-deficient human serum and stained. *a*, FITC-goat antihuman Clq; *b*, FITC-goat antihuman C3. Positive staining (*speckles*) on the surface of the cells is evident in *a*, but not *b*. Original magnification × 625.

TABLE I. *In vitro complement staining reactions with mouse epidermal monolayers and organ cultured normal human skin*

	Complement treatment[a]	Reaction[b]		
		Clq	C4	C3
Pemphigus IgG	No C	–	–	–
	C	++	+++	+++
	Purified Clq	++	–	–
	C 56°C for 30 min	–	–	–
	C plus EDTA	+	–	–
	C plus EGTA-Mg++	+	+	–
	C2-deficient serum	++	++	–
	C plus anti-C3	++	++	–
Normal human IgG	No C	–	–	–
	C	–	–	–
	C2-deficient serum	–	–	–
Saline	C	–	–	–

[a] C: normal human serum (complement source).
[b] +: speckled pattern in epidermal monolayer cells or intercellular staining in organ cultured skin and mucosa. Results obtained using epidermal monolayer cells or organ cultued skin were identical.

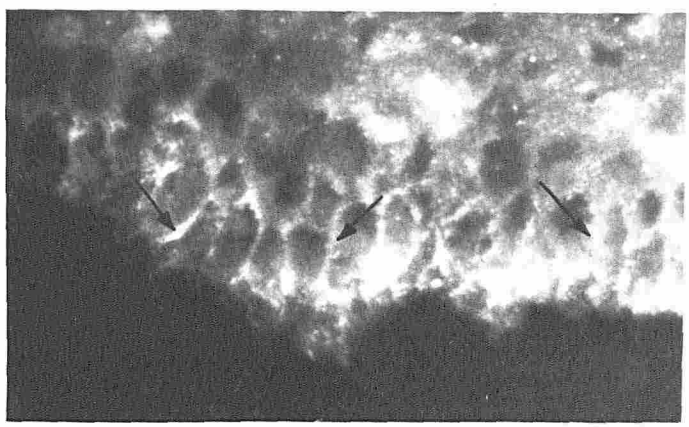

FIG 3. Forty-eight-hour adult human skin organ culture in heat-inactivated normal human serum containing 6.75 mg/ml pemphigus IgG. *Arrows* indicate C3 staining reaction in the intercellular areas. The epidermis depicted has separated from the basal cells and the dermis. Original magnification × 500.

phigus antibodies are capable of activating complement.

As with bullous pemphigoid antibodies [17,18] and the factor found in herpes gestationis sera [19], the major pathway activated by pemphigus antibody is the classical pathway. Significant in vitro binding of Clq and C4 by pemphigus antibody was noted in both organ culture and tissue culture skin in addition to C3. These in vitro findings also support previous direct IF studies where these same components have been found bound to ICS areas in pemphigus skin lesions [7,8]. Blockage of the classical pathway by heat inactivation or addition of Mg++-EGTA to the complement source, or by substituting C2-deficient serum as the complement source, inhibited the positive C3 staining.

In addition to direct IF studies, other findings have implicated the complement system in the pathogenesis of pemphigus. Low levels of total hemolytic complement and individual complement components have been noted in blister fluids of pemphigus patients, suggestive of local activation [20]. High-molecular-weight anticomplementary activity [21] and Clq binding activity [22] have also been detected in pemphigus sera and blister fluids, suggestive of immune complex formation. Further, cryoproteins, which contain IgG with pemphigus antibody and complement components, have been identified in sera of clinically active cases [23].

The specific role that complement plays, if any, in the pathogenesis of pemphigus is presently unknown. Clearly, pemphigus antibody alone is sufficient to initiate the process of acantholysis in vitro by organ culture methods [16] and to cause detachment of epidermal cells in culture and release of plasminogen activator [24,25]. Whether these processes are accelerated in the presence of complement must still be determined. These and other questions concerning the involvement of the

complement system in pemphigus are currently being investigated.

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